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2 # Interference of Neuronal TrkB Signaling by the Cannabis-Derived Flavonoids

3 # Cannflavins A and B

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15 **ABSTRACT**

16 Cannflavins A and B are two flavonoids that accumulate in the *Cannabis sativa* plant. These
17 specialized metabolites are uniquely prenylated and highly lipophilic, which, *a priori*, may permit
18 their interaction with membrane-bound enzymes and receptors. Although previous studies found
19 that cannflavins can produce anti-inflammatory responses by inhibiting the biosynthesis of pro-
20 inflammatory mediators, the full extent of their cellular influence remains to be understood. Here,
21 we studied these flavonoids in relation to the Tropomyosin receptor kinase B (TrkB), a receptor
22 tyrosine kinase that is activated by the growth factor brain-derived neurotrophic factor (BDNF).
23 Using mouse primary cortical neurons, we first collected evidence that cannflavins prevent the
24 accumulation of Activity-regulated cytoskeleton-associated (Arc) protein upon TrkB stimulation
25 by exogenous BDNF in these cells. Consistent with this effect, we also observed a reduced
26 activation of TrkB and downstream signaling effectors that mediate *Arc* mRNA transcription when
27 BDNF was co-applied with the cannflavins. Of note, we also performed a high-throughput screen
28 that demonstrated a lack of agonist action of cannflavins towards 320 different G protein-coupled
29 receptors, a result that specifically limit the possibility of a TrkB transinactivation scenario via G
30 protein signaling to explain our results with dissociated neurons. Finally, we used Neuro2a cells
31 overexpressing TrkB to show that cannflavins can block the growth of neurites and increased
32 survival rate produced by the higher abundance of the receptor in this model. Taken together, our
33 study offers a new path to understand the reported effects of cannflavins and other closely related
34 compounds in different cellular contexts.

35 **INTRODUCTION**

36 Flavonoids are polyphenolic compounds found in various plant-derived foods and beverages.
37 These phytochemicals represent a large family of molecules that can be classified into six main
38 subclasses, based on their chemical structure: flavonols, flavanols (also known as flavan-3-ols or
39 catechins), flavanones, flavones, anthocyanins, and isoflavones (Panche et al., 2016). Interestingly,
40 evidence suggests that moderate habitual intake of flavonoids can lower the risk of cardiovascular
41 disease, cancer, as well as all-cause mortality (Bondonno et al., 2019). Another purported benefit
42 of these natural products is their positive influence on brain health and function, as several
43 members of the flavonoid family have been found to promote neuroprotection, reduce
44 neuroinflammation, and enhance cognition (Vauzour et al., 2008; Jaeger et al., 2018; Bakoyiannis
45 et al., 2018). More precisely, flavonoids appear to modulate signaling pathways that are central to
46 the control of neuronal survival and plasticity, such as the MAPK-CREB and PI3K-mTOR
47 cascades (Vauzour et al., 2008; Jaeger et al., 2018). However, this particular line of inquiry has
48 been little studied and therefore represents an opportunity to identify new bioactive compounds
49 with therapeutic qualities among this family of phytochemicals.

50 Apart from the psychoactive molecule Δ^9-tetrahydrocannabinol (THC) and other related
51 cannabinoids with only mild or no psychotropic effect, like cannabidiol (CBD) and cannabigerol
52 (CBG), the *Cannabis sativa (C. sativa)* plant also produces hundreds of specialized metabolites
53 including at least twenty different flavonoid compounds (Flores-Sanchez et al., 2008). Among
54 those, the flavones cannflavin A and cannflavin B (Figure 1A) are considered to accumulate
55 uniquely in *C. sativa* cultivars. Seminal work by Barrett and colleagues performed more than 30
56 years ago helped identify these two flavonoids and characterize them as inhibitors of prostaglandin
57 E_2 production with the ability to produce anti-inflammatory effects that are approximately thirty

58 times more potent than acetylsalicylic acid, better known as aspirin (Barrett et al., 1985; 1986).
59 However, a broader understanding of cannflavins' influence on cell biology in health and disease
60 did not progress much since their initial description because of challenges associated with their
61 extraction and the various political landscapes that limited their distribution. Nevertheless, some
62 pre-clinical studies have provided intriguing new details about these molecules in recent years. For
63 instance, the unnatural isomer of cannflavin B named FBL-03G (also known as caflanone) was
64 found to increase apoptosis of pancreatic cancer cells *in vitro* while animal tests showed that the
65 same small molecule could limit progression of metastatic pancreatic cancer (Moreau et al., 2019).
66 Additionally, another study reported a possible neuroprotective effect of cannflavin A at
67 concentrations lower than 10 μM that was attributed to the ability of this molecule to reduce
68 aggregation of β amyloid through direct docking in the hydrophobic groove of the protein (Eggers
69 et al., 2019). While these different findings provide novel insights about the pharmacological
70 potential of cannflavins, the full range of molecular changes induced by cannflavins in cells
71 remains to be described. To address this gap in our understanding of cannflavin pharmacology, we
72 therefore focused on identifying novel mechanisms of action of these two related cannabis-derived
73 metabolites in neuronal cells.

74 Cannflavins A and B are prenylated and highly lipophilic small molecules (Barrett et al., 1985;
75 Choi et al., 2004), a characteristic that allows them to easily accumulate into cells where they can
76 then presumably interact with different membrane-bound enzymes and receptors. Previously, we
77 published a chemogenomic analysis that aimed at identifying small molecule modulators of
78 Activity-regulated cytoskeleton-associated protein (Arc), which is a key regulator of
79 neuroplasticity and cognitive functions (Bramham et al., 2010; Korb et al., 2011; Kedrov et al.,
80 2019). Our approach in that project exploited the ability of the growth factor brain-derived

81 neurotrophic factor (BDNF) to promote abundant *Arc* mRNA expression followed by nuclear
82 accumulation of the protein product in mouse primary cortical neurons via activation of
83 Tropomyosin receptor kinase B (TrkB) receptor (Lalonde et al., 2017). Here, we have adapted this
84 assay to test the two cannflavins and found evidence of TrkB signaling interference by both
85 molecules. These results then led us to complete a secondary high-throughput screen to test
86 possible agonist activity of these flavonoids on G protein-coupled receptors (GPCRs), as well as
87 other biochemical assays to confirm the influence of cannflavins and pinpoint their potential target
88 engagement. These specific efforts suggest a model where cannflavins interfere with TrkB activity
89 through direct inhibitory action on the receptor. Finally, image-based cellular test with
90 immortalized Neuro2a cells ectopically expressing TrkB allowed us to demonstrate the capacity
91 of cannflavins to block BDNF-induced neurite outgrowth. In summary, our study supports the
92 classification of cannflavins as candidate inhibitors of TrkB receptor signaling in neuronal cells.
93

94 **METHODS**

95 *Cell culture and transfection*

96 Developing cerebral cortex from E16.5 CD-1 mouse embryos were dissected and then dissociated
97 in trypsin solution for 15 min followed by three washes with phosphate-buffered saline (PBS).
98 Trypsinized tissue was gently triturated to produce single cell suspension. Next, cells were seeded
99 in poly-L-lysine/laminin coated 6-well plates at a density of 1.5×10^6 per well and maintained in
100 Neurobasal medium containing B27 supplement (2%, Invitrogen, Grand Island, NY), penicillin
101 (50 U/ml, Invitrogen), streptomycin (50 µg/ml, Invitrogen) and glutamine (1 mM, Sigma). For
102 experiments involving BDNF (PeproTech, Rocky Hill, NJ), the growth factor was added directly
103 to the culture medium at a final concentration of 100 ng/ml for the indicated period of time.

104 Preparation of mouse primary cortical neuron cultures was approved by the University of Guelph
105 Animal Care Committee and carried out according to institutional guidelines.
106 For neurite outgrowth assay, Neuro2a cells were cultured in DMEM [supplemented with 10%
107 HyClone FetalClone II serum (Cytiva, Global Life Sciences Solutions, Marlborough, MA),
108 penicillin (50 units/ml), and streptomycin (50 μg/ml)] and transfected overnight using
109 Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.
110
111 *Antibodies, plasmid, and pharmacological compounds*
112 The anti-Arc rabbit polyclonal affinity purified antibody (#156 003) was purchased from Synaptic
113 Systems (Goettingen, Germany). The antibodies recognizing p42 Mapk (Erk2, sc-1647) was from
114 Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies recognizing phosphorylated
115 $TrkA^{Tyr490}$/$TrkB^{Tyr516}$ (#4619), phosphorylated p44/42 Mapk ($Erk1/2^{Thr202/Tyr204}$, #4370), Akt
116 (#4691), phosphorylated Akt^{Thr308} (#2965), phosphorylated Akt^{Ser473} (#4060), mTor (#2983),
117 phosphorylated $mTor^{Ser2448}$ (#2971), and phosphorylated $rpS6^{Ser240/244}$ (#2215) were acquired from
118 Cell Signaling Technology (Beverly, MA). The antibodies recognizing TrkB (MAB397) were
119 acquired from R&D Systems (Minneapolis, MN). The antibodies recognizing β-actin (A1978) and
120 M2 FLAG (F1804) antibodies were from Sigma-Aldrich (St-Louis, MO), while the Map2
121 (AB5543) antibody was purchased from EMD Millipore Corps (Billerica, MA). Finally, cross-
122 absorbed horseradish peroxidase-conjugated secondary antibodies were from Invitrogen.
123 The pCMV6-Ntrk2-Myc-DDK (FLAG) plasmid (MR226130) was purchased from OriGene
124 Biotechnologies (Rockville, MD). ECGC, genistein, and daidzein were from Sigma-Aldrich.
125 ANA-12 (Figure 1B) was from Tocris Bioscience (Bristol, UK) and U0126 was from Biosciences
126 (Thermo Fisher).

127 The synthesis and purification of cannflavins A and B were produced using the method of Rea
128 and colleagues (2019). Briefly, *Cannabis sativa* L. prenyltransferase 3 (CsPT3) was recombinantly
129 expressed in *Saccharomyces cerevisiae* and the microsomal fraction containing CsPT3 was
130 collected for *in vitro* enzyme assays. Assays containing 200 μM chrysoeriol, 400 μM GPP or
131 DMAPP, 1 mg/mL of microsomal protein, and 10 mM $MgCl_2$ in 100 mM Tris-HCl buffer were
132 conducted at 37ºC for 120 min and terminated with the addition of 20% formic acid. Cannflavin
133 products were extracted with three volumes of ethyl acetate, the organic layer was dried under N_2
134 gas and resuspended in methanol. The products were purified by HPLC on an Agilent 1260 Infinity
135 system equipped with a Waters SPHERISORB 5 μm ODS2 column, and eluted with a 20 min
136 linear gradient from 45% to 95% methanol in water containing 0.1% formic acid. Product identities
137 were confirmed via LC-MS according to published methods (Rea et al., 2019) (Supplementary
138 Figure 1). Cannflavins produced *in vitro* were dried under nitrogen gas and resuspended in
139 dimethyl sulfoxide (DMSO). The final products were confirmed via HPLC, as described above,
140 and quantified by absorption at 340 nm relative to authentic standards.
141
142 *Western blotting*
143 For western blot analyses, cells were collected by scraping in ice-cold radioimmunoprecipitation
144 assay (RIPA) buffer (50 mM tris-HCl [pH 8.0], 300 mM NaCl, 0.5% Igepal-630, 0.5%
145 deoxycholic acid, 0.1% SDS, 1 mM EDTA) supplemented with a cocktail of protease inhibitors
146 (Complete Protease Inhibitor without EDTA, Roche Applied Science, Indianapolis, IN) and
147 phosphatase inhibitors (Phosphatase Inhibitor Cocktail 3, Sigma-Aldrich). One volume of 2×
148 Laemmli buffer (100 mM tris-HCl [pH 6.8], 4% SDS, 0.15% bromophenol blue, 20% glycerol,
149 200 mM β-mercaptoethanol) was added and the extracts were boiled for 5 min. Samples were

150 adjusted to an equal concentration after protein concentrations were determined using the BCA
151 assay (Pierce, Thermo Fisher Scientific). Lysates were separated using SDS–PAGE
152 (polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane. After transfer,
153 the membrane was blocked in TBST (tris-buffered saline and 0.1% Tween 20) supplemented with
154 5% nonfat powdered milk and probed with the indicated primary antibody at 4°C overnight. After
155 washing with TBST, the membrane was incubated with the appropriate secondary antibody and
156 visualized using enhanced chemiluminescence (ECL) reagents according to the manufacturer's
157 guidelines (Pierce, Thermo Fisher Scientific).

158 The following procedure was used to quantify western blot analyses. First, equal quantity of
159 protein lysate as determined by the BCA assay was analyzed by SDS-PAGE for each biological
160 replicate. Second, the exposure time of the film to the ECL chemiluminescence was the same for
161 each biological replicate. Third, all the exposed films were scanned on a HP Laser Jet Pro M377dw
162 scanner in grayscale at a resolution of 300 dpi. Fourth, the look-up table (LUT) of the scanned tiff
163 images was inverted and the intensity of each band was individually estimated using the selection
164 tool and the histogram function in Adobe Photoshop CC 2021 software. Finally, the intensity of
165 each band was divided by the intensity of its respective loading control (β-actin) to provide the
166 normalized value used for statistical analysis.

167

168 *Immunocytochemistry*

169 Indirect immunofluorescence detection of antigens was carried out using cortical neurons cultured
170 on poly-L-lysine/laminin coated coverslips in 24-well plates at a density of 0.1×10^6 per well.
171 After experimental treatment, cells were washed twice with phosphate-buffered saline (PBS) and
172 fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. After fixation, cells were

173 washed twice with PBS, permeabilized with PBST (PBS and 0.25% Triton X-100) for 20 min,
174 blocked in blocking solution (5% goat non-immune serum in PBS) for another 30 min, and finally
175 incubated overnight at 4°C with the first primary antibody in blocking solution. The next day,
176 coverslips were extensively washed with PBS and incubated for 2 hours at room temperature in
177 the appropriate fluorophore-conjugated secondary antibody solution [Alexa Fluor 488-, Alexa
178 Fluor 594, or Alexa Fluor 647-conjugated secondary antibody (Molecular Probes, Invitrogen) in
179 blocking solution]. After washes with PBS, the coverslips were incubated again overnight in
180 primary antibody solution for the second antigen, and the procedure for conjugation of the
181 fluorophore-conjugated secondary antibody was repeated as above. Finally, cell nuclei were
182 counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and coverslips were mounted on glass
183 slides with ProLong Antifade reagent (Invitrogen).

184 Cells cultured on coverslips from three independent biological replicates were imaged with a
185 Nikon Eclipse Ti2-E inverted microscope equipped with a motorized stage, image stitching
186 capability, and a 60× objective (Nikon Instruments, Melville, NY). Image analysis was performed
187 with ImageJ and NIS Elements and the following procedure was used to quantify nuclear Arc level
188 in response to BDNF-TrkB signaling. First, original raw tiff files were opened and the nucleus of
189 all neurons in the image was located based on Map2 immunostaining, then average pixel intensity
190 corresponding to Arc immunofluorescence was measured for a 30-pixel spot positioned at the
191 center of the nuclear compartment. Second, for each measure of Arc nuclear immunofluorescence
192 pixel intensity, a measure of background pixel intensity from the same image channel was acquired
193 and subsequently subtracted from the Arc nuclear immunofluorescence pixel intensity value.
194 Finally, Arc immunofluorescence signal from untreated samples was used to establish an objective
195 threshold (two standard deviations above the nuclear Arc immunofluorescence signal averaged

196 from a representative population of untreated neurons) which allowed for comparison of nuclear
197 Arc expression between different experimental conditions.
198
199 *Real-time reverse transcriptase PCR*
200 After experimental treatment, total RNA was isolated from primary cortical neuron cultures using
201 the TRIzol method (Invitrogen). The concentration of total RNA was measured using a NanoDrop
202 ND-8000 spectrophotometer (Thermo Fisher Scientific) and first-strand complementary DNA
203 (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-
204 time PCRs were performed using gene-specific primers and monitored by quantification of SYBR
205 Green I fluorescence using a Bio-Rad CFX96 Real-Time Detection System. Expression was
206 normalized against *Gapdh* expression. The relative quantification from three biological replicates
207 was calculated using the comparative cycle threshold ($\Delta\Delta C_T$) method.
208 Primers for real-time reverse transcription PCR experiments were: *Arc* primer pair one, 5′-
209 TAGCCAGTGACAGGACCCAG-3′ (forward) and 5′-CAGCTCAAGTCCTAGTTGGCAAA-3′ (reverse);
210 *Arc* primer pair two, 5′- CGCCAAACCCAATGTGATCCT-3′ (forward) and 5′-
211 TTGGACACTTCGGTCAACAGA-3′ (reverse); *Gapdh*, 5′-ATGACCACAGTCCATGCCATC-3′ (forward)
212 and 5′-CCAGTGGATGCAGGGATGATGTTC-3′ (reverse).
213
214 *PRESTO-Tango GPCR assay*
215 Parallel receptorome expression and screening via transcriptional output, with transcription
216 activation following arresting translocation (PRESTO-Tango) was used to assess cannflavin A and
217 cannflavin B potential to stimulate G protein-coupled receptors (GPCRs) according to published
218 methods (Kroeze et al., 2015). Overall, 320 distinct nonolfactory human GPCRs were tested.
219

220 *Neurite outgrowth assessment*

221 Neuro2A cells transfected with a pCMV6-Ntrk2-Myc-DDK (FLAG) construct were selected with
222 G-418 (Geneticin) to produce a stable cell line that constitutively expresses Myc-FLAG tagged-
223 TrkB. For neurite outgrowth assessment, cells were seeded on 15 mm glass coverslips in 12-well
224 plates at a density of 2.0×10^4 per well and allowed to attach overnight. Next day, cells were treated
225 with recombinant BDNF (1 nM) plus cannflavins (10 μM), ANA-12 (10 μM), or vehicle control
226 (DMSO). Phase contrast digital images were collected with a Nikon Eclipse Ti2-E inverted
227 microscope and a 20× objective 24 hours after start of treatment (five fields per dish, three wells
228 per condition). Image analysis was completed using ImageJ software using the following
229 procedure. First, any cells with a fragmented nucleus were excluded from the analysis, therefore
230 the total number of viable cells was counted per field. Second, for all identified viable cells the
231 total number of neurites and number of cells with neurites longer than 2 cell bodies in diameter
232 were counted per field.

233

234 *Statistics*

235 Unless mentioned otherwise, all results represent the mean ±SEM from at least three independent
236 experiments. ANOVA followed by Tukey's or Dunnett's post hoc test for multiple comparisons
237 were performed where indicated.

238

239 **RESULTS**

240 *Impact of cannflavins on BDNF-induced Arc expression in mouse primary cortical neurons*

241 Previously, we completed a chemogenomic screen with primary mouse cortical neurons that
242 identified a suite of compounds that acted as Arc expression modifiers (Lalonde et al., 2017). Part

243 of this group included five distinct flavonoids (Figure 1C)—namely (–)-epigallocatechin (ECGC),
244 baicalin (BAI), 7,8-dihydroxyflavone (7,8-DHF), daidzein, and genistein—which were found to
245 enhance nuclear Arc protein levels above the control measure when co-applied at a final
246 concentration of 16.7 μM with recombinant BDNF for 6 h. Searching for a possible explanation
247 to this phenomenon, we were intrigued by several studies that had linked each of these five
248 flavonoids to either enhancement of *BDNF* and/or *TrkB* mRNA expression, or to the potentiation
249 of downstream TrkB-dependent signaling (Pan et al., 2012; Gundimeda et al., 2014; Ding et al.,
250 2018; Lu et al., 2019). Based on these observations, we hypothesized that cannflavin A and
251 cannflavin B could act in a similar fashion and promote Arc protein abundance when added to
252 cultured mouse cortical neurons that were stimulated with exogenous BDNF. Unexpectedly,
253 though, western blot analysis assessing BDNF-induced Arc expression in conjunction with
254 cannflavins with concentrations of the flavonoids ranging between 1 to 20 μM revealed an opposite
255 effect. Specifically, we found that application of cannflavins to cell culture media prevented the
256 normal increase in Arc protein by BDNF in a dose-dependent manner where 10-20 μM of
257 cannflavin A, and all tested concentrations (1-20 μM) of cannflavin B, resulted in significantly
258 less Arc protein abundance than seen in the BDNF-alone control measure (Figure 2A). To further
259 support this result, we repeated the experiment using fluorescent immunocytochemistry and
260 quantified nuclear Arc changes, as we had done previously in our chemogenomic screen (Lalonde
261 et al., 2017). Of note, we also tested the flavonol ECGC, as well as the isoflavone daidzein and
262 genistein, since those molecules were found to produce the opposite effect of increasing BDNF-
263 induced nuclear Arc levels according to our previous screening results. As shown in Figure 2B
264 and Supplementary Figure 2, cells that were co-treated with BDNF and 10 μM daidzein or
265 genistein presented a moderate increase in the percentage of nuclei with Arc expression above

266 threshold in comparison to the BDNF-alone control. Interestingly, though, the ECGC condition
267 was similar on average to the BDNF-alone condition suggesting that the final concentration of this
268 specific flavonoid must be greater than 10 μM to have an impact of TrkB-induced nuclear Arc
269 level. Most importantly, and consistent with our western blot analysis above, cultures treated with
270 BDNF and 1-10 μM cannflavins presented similar overall trends in the reduction of Arc-positive
271 neuronal abundance in comparison to the unstimulated control (Figure 2C). Together, these results
272 confirm the observed discrepancy between cannflavins and the other flavonoids found in our
273 earlier screen, which focused on BDNF-induced Arc expression modifiers.

274 Next, to ascertain whether the cannflavins' influence on TrkB signaling and Arc protein levels
275 was produced before or after gene transcription, we performed a quantitative polymerase chain
276 reaction (qPCR) experiment using two pairs of primers targeting different regions of the *Arc*
277 transcript. Here, comparison of *Arc* mRNA abundance between untreated, BDNF-alone control,
278 and BDNF with cannflavin A (10 μM) or cannflavin B (10 μM) samples clearly indicated that
279 cannflavins prevent induction of *Arc* mRNA expression (Figure 2D), therefore suggesting that the
280 effect of these compounds must occur somewhere between the activation of TrkB receptors by
281 BDNF and the activation of the transcriptional machinery involved in *Arc* expression.

282

283 *Evaluating agonist potential of cannflavins on Tango GPCR assay*

284 Considering the transactivation crosstalk between GPCRs and receptor tyrosine kinases, including
285 TrkB (Rajagopal et al., 2004; 2006; El Zein et al., 2007), and recent evidence for GPCR
286 modulation/self-association by flavonoids on the latter (Herrera-Hernández et al., 2017; Ortega et
287 al., 2019), we speculated that one mechanism by which cannflavins could interfere on *Arc* mRNA
288 expression in cortical neurons involves activation of a G protein signal that transinactivates the

289 function of molecular cascades downstream of TrkB receptors responsible for *Arc* expression. To
290 explore this scenario, we tested the effect of cannflavins on the GPCRome, *en masse,* using the
291 PRESTO-Tango assay—an unbiased high-throughput screening approach adapted to identify
292 agonist activity of agents towards the large family of GPCRs (Kroeze et al, 2015). Interestingly,
293 applying cannflavin A revealed no effect on any of the 320 different GPCRs tested while
294 cannflavin B was found to produce only weak increase (4.4 fold-change) of GPR150 activity from
295 baseline, a negligible effect in comparison to the positive control (51.3 fold-change, dopamine D_2
296 receptor stimulated by quinpirole) (Figure 3). Faced with these results, we then re-focused our
297 attention on the possibility that cannflavins act more directly on the TrkB receptor and/or its
298 downstream signaling components.
299
300 *Elucidating cannflavins effects on TrkB signaling*
301 BDNF binding to the extracellular domain of a TrkB receptor stimulates its dimerization and the
302 phosphorylation of intracellular tyrosine residues which is followed by the recruitment of
303 pleckstrin homology (PH) and SH2 domain containing proteins—such as FRS2, Shc, SH2B, and
304 SH2B2—that regulate distinct concurrent signaling cascades (Qian et al., 1998; Meakin et al.,
305 1999). To explore whether cannflavins interfere with the activation of TrkB receptors by BDNF
306 in primary cortical neurons, we used a western blotting approach and probed lysates with a P-
307 TrkA/B antibody. This approach revealed that, indeed, cannflavins can prevent BDNF from
308 effectively stimulating its target receptor (Figure 4A). To further support this result, we tested the
309 activation of signaling pathways that are likely regulated downstream of TrkB, including the Ras-
310 Raf-MEK-Mapk and the PI3K/Akt/mTor cascades (Huang et al., 2003; Kowiański et al., 2018)
311 (Figure 4B). Interestingly, our analyses revealed that both cannflavin A and cannflavin B sharply

312 reduced normal increase in P-Mapk, P-Akt, P-mTor, and P-rpS6 levels produced by BDNF (Figure
313 4C). The fact that there were those changes in these three molecular pathways, which to a great
314 extent occurs in parallel with limited cross-interaction (Kowiański et al., 2018), strongly suggest
315 that cannflavins must act at an early stage in TrkB signal activation.
316
317 *Functional characterization TrkB inhibition by cannflavins on BDNF-dependent neurite*
318 *outgrowth*
319 Our biochemical analyses with mouse primary cortical neurons suggest that cannflavins A and B
320 have inhibitory activity towards TrkB receptors. In order to establish if this effect is sufficient to
321 limit cellular processes under the control of BDNF signaling, we used neuroblastoma Neuro2a
322 cells stably expressing Ntrk2 (TrkB)-Myc-FLAG to complete a neurite outgrowth experiment
323 (Figure 5A). As shown in Figure 5B, Neuro2a cells have low TrkB expression with negligeable
324 phosphorylation of the receptor under basal conditions, while cells stably expressing the receptor
325 display greater responsiveness to exogenous application of BDNF—which is clearly demonstrated
326 by higher level of P-TrkB. Further, pre-application of cannflavins (20 μM) with BDNF to the
327 culture media for 6 h reduced BDNF-induced TrkB phosphorylation (Figure 5C). Interestingly, we
328 did not observe a decrease in P-Mapk levels with treatment of ANA-12 or cannflavins as was
329 observed in cortical neurons (Figure 4C), a distinction that we attribute to the fact that
330 overexpression of TrkB in Neuro2A produced maximal phosphorylation of the p-42 subunit of
331 Mapk and no change in signal when BDNF was added to the Neuro2a cells (Figure 5B). Most
332 importantly, neurite assays completed with and without the application of ANA-12, cannflavin A,
333 and cannflavin B revealed that all three compounds produced a significant decrease in the total
334 number of neurites per field (Figure 5D and E) and number of cells with processes twice the length

335 of the cell (Figure 5F) when applied at a final concentration of 10 µM to culture media. We also
336 noticed a significant decrease in viable cells (Figure 5G) at this same concentration but only
337 between the BDNF control condition and the BDNF plus ANA-12 or cannflavins. Of note, our
338 measures of total number and length of neurites, as well as cell survival, for the ANA-12 and
339 cannflavins conditions in this experiment were comparable to the baseline values were observed
340 in wild-type Neuro2a cells not overexpressing TrkB (Supplementary Figure 3). Altogether, our
341 results here strongly support that cannflavins act on TrkB receptors, preventing BDNF activation
342 of downstream signaling of the receptor.
343

344 **DISCUSSION**

345 This study provides evidence for an inhibitory effect of cannflavins A and B, two flavonoids from
346 *C. sativa*, on BDNF-induced Arc expression through disruption of TrkB receptor signaling. These
347 results contrast our previous observations that various flavonoids exhibit a potentiating effect
348 towards BDNF-induced Arc accumulation in mouse primary cortical neurons (Lalonde et al.,
349 2017). Strikingly, the addition of cannflavin A or cannflavin B to the culture medium of mouse
350 primary cortical neurons at a minimum concentration of 5 µM consistently prevented the induction
351 of *Arc* mRNA expression, suggesting that these molecules act between BDNF-activation of TrkB
352 receptors and transcription of the *Arc* gene. Therefore, we subsequently investigated the impact of
353 cannflavins on the downstream pathways of TrkB using biochemical analyses and uncovered a
354 consistent decrease in the activation of the Ras-Raf-Mek-Mapk and the PI3K/Akt/mTor cascades.
355 In connection with these results, we demonstrated that cannflavins inhibited BDNF-induced
356 neurite outgrowth in neuroblastoma Neuro2a cells stably overexpressing TrkB. Taken together,
357 our study provides a new path to better understand the effects that have been reported in recent

358 years about cannflavins and other closely related compounds against certain cancer cell types
359 (Brunelli et al., 2009; Moreau et al., 2019).
360
361 *Structural determinants of cannflavins activity towards TrkB*
362 All flavonoids have a basic flavan nucleus with two aromatic rings (the A and the B rings)
363 interconnected by a three-carbon-atom heterocyclic ring (the C ring), as illustrated in Figure 1C
364 for the flavone 7,8-dihydroxyflavone (7,8-DHF, also known as tropoflavin). Interestingly, a
365 previous study focusing on 7,8-DHF, which is a compound reported to mimic the physiological
366 activity of BDNF and stimulate TrkB signaling *in vitro* and *in vivo* (Jang et al., 2010; Zeng et al.,
367 2012), helps speculate about what precise structural feature might confers TrkB antagonistic
368 activity to cannflavins. Specifically, previous comparison of different 7,8-DHF derivatives on
369 TrkB phosphorylation and downstream Akt signaling revealed that the presence of a 3'-hydroxy
370 group (or to a lesser extent a 2'-hydroxy group) on the B ring confers a TrkB stimulatory effect to
371 a 7,8-DHF derivative compound whereas addition of a 4'-hydroxy group, as shown in Figure 6A
372 with 7, 8, 4'-trihydroxyflavone and Figure 6B with 3, 5, 7, 8, 3, 4'-hexahydroxyflavone, inversely
373 mediates inhibition of the receptor (Liu et al., 2010). Since cannflavins A and B are both
374 hydroxylated at the 4' position on the B ring (Figure 1A), similar to compounds found by Liu and
375 colleagues (2010) interfering with TrkB phosphorylation in rat primary cortical neurons (Figures
376 6A and 6B), we thus suspect that this specific structural feature is key in mediating the TrkB
377 antagonistic effect observed in our study. In this context, it will be revealing whether cannflavin
378 derivatives with different patterns of hydroxylation on the B ring produce a different activity
379 towards TrkB and downstream cellular effects.

380 In addition to 4' hydroxylation of the B ring, prenylation is another structural element that we
381 must consider in relation to the difference we consistently observed between cannflavin A and
382 cannflavin B towards TrkB. As seen in Figure 1A, cannflavin B has only one isoprene unit while
383 cannflavin A has two, making the later an overall larger and more lipophilic molecule.
384 Consequently, we speculate that the smaller size of cannflavin B may facilitate access and/or create
385 a stronger binding affinity to TrkB in a cellular context, which could then explain why we have
386 been measuring a more potent inhibitory response of the receptor phosphorylation and blunting of
387 downstream signaling with this specific compound. Here, though, we also need to acknowledge
388 the fact that our data do not demonstrate direct interaction of cannflavins A and B with TrkB at
389 this point. Nevertheless, two specific results from our study support to a certain degree the idea
390 that a direct functional interaction most likely occur between cannflavins and TrkB. First, our
391 interrogation of the GPCRome using the PRESTO-Tango assay showed that cannflavins do not
392 stimulate the activity of more than 300 GPCRs, which rules out the possibility that cannflavins are
393 disrupting TrkB function through a GPCR transinactivation event in our cellular experiments
394 (Rajagopal et al., 2004; 2006; El Zein et al., 2007). And second, our experiment with Neuro2a
395 cells stably expressing TrkB reveals that cannflavins block the growth of neurites, as well as the
396 observed cell survival stimulatory effect, produced by exogenous BDNF application in this model.
397 Since these phenotypes are directly tied to the overexpression of TrkB, and that application of
398 cannflavins consistently returns neurite and survival measures to those of wild-type Neuro2a cells
399 (Supplementary Figure 3), we consider these results as evidence for a direct interaction between
400 cannflavins and TrkB. To conclude on this point, whether the activity of receptor tyrosine kinases
401 other than TrkB is interfered by cannflavins remains unknown and should be considered in future
402 research.

403 *Therapeutic potential of cannflavins*

404 Although cannflavins are recognized to produce potent anti-inflammatory effects by inhibiting the
405 biosynthesis of various pro-inflammatory mediators, including microsomal prostaglandin E_2
406 synthase-1 (mPGES-1) and 5-lipoxygenase (5-LO) (Barrett et al., 1985; 1986; Werz et al., 2014),
407 our study cautions that these flavonoids may not be best to intervene against neuroinflammation
408 or provide pro-cognitive effects because of their impact on TrkB signaling (Jaeger et al., 2018).
409 That being said, cannflavin A and/or cannflavin B may prove to be helpful in other circumstances
410 where TrkB signaling is instead found to be overactive or dysregulated. For instance, increased
411 TrkB expression was detected in low-grade astrocytoma and glioblastoma (Wadhwa et al., 2003;
412 Assimakopoulou et al., 2007), while BDNF-induced activation of TrkB has been found to increase
413 the viability of brain-tumor stem cells isolated from glioblastoma (Lawn et al., 2015). Furthermore,
414 a study uncovered a link between the ability of glioblastoma to make less invasive cancer cells
415 around them more aggressive via the transfer of TrkB-containing exosomes, revealing this way a
416 mechanism by which these tumours can influence their environment to promote disease
417 progression and aggressiveness (Pinet al et., 2016), while other reports have shown that inhibition
418 of TrkB-associated signaling may be an effective strategy to limit the formation of astrocytomas
419 (Ni et al., 2017) as well as the survival of glioblastoma cancer cells (Pinheiro et al., 2017). Finally,
420 emerging evidence suggest that TrkB signaling could also be a therapeutic target for other cancer
421 types, including lung (Sinkevicius et al., 2014; Chen et al., 2016), breast (Choy et al., 2017;
422 Contreras-Zárate et al., 2019), and pancreatic cancer (Oyama et al., 2021). Taken together, these
423 studies suggest that targeting TrkB signaling with cannflavins may provide therapeutic benefits
424 against these cancer types. In conclusion, our study expands the range of cellular effect for

425 cannflavins beyond inflammation and supports the examination in more detail of these compounds
426 as possible anti-cancer agents.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The use of mice for primary neuron cultures was reviewed and approved by the University of Guelph Animal Care Committee.

CONFLICT OF INTEREST

TAA received sponsored research funding from Atlas 365 that was used for the preparation of cannflavins used in this research. The other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JH, AWM, BA, JYK, and JL planned and designed the experiments. CP, JYK, and TAA provided key resources, and JH, AWM, BA, and CA performed the research. JH, AWM, BA, CP, CA, and JL analyzed the data and prepared figures. JH, AWM, and JL wrote the manuscript with revisions from all other authors.

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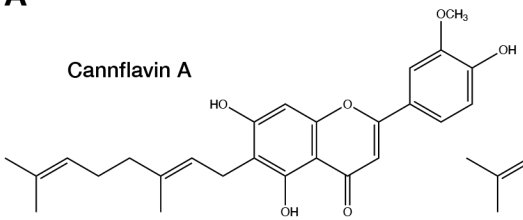
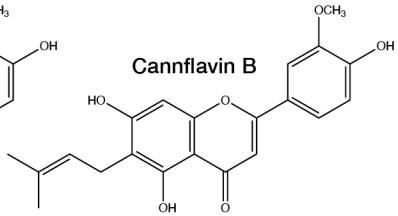
A

Cannflavin A

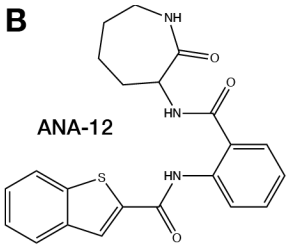


Cannflavin B

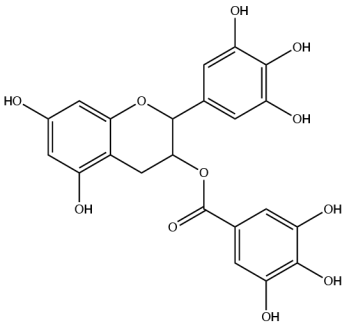
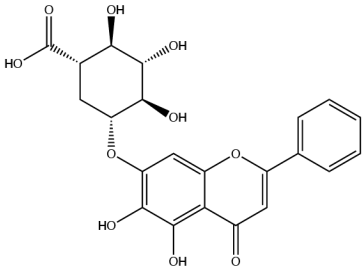
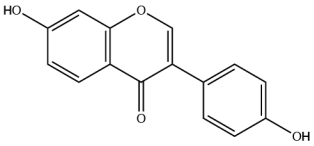
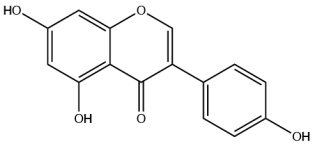
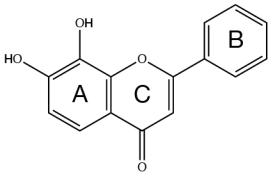


B

ANA-12

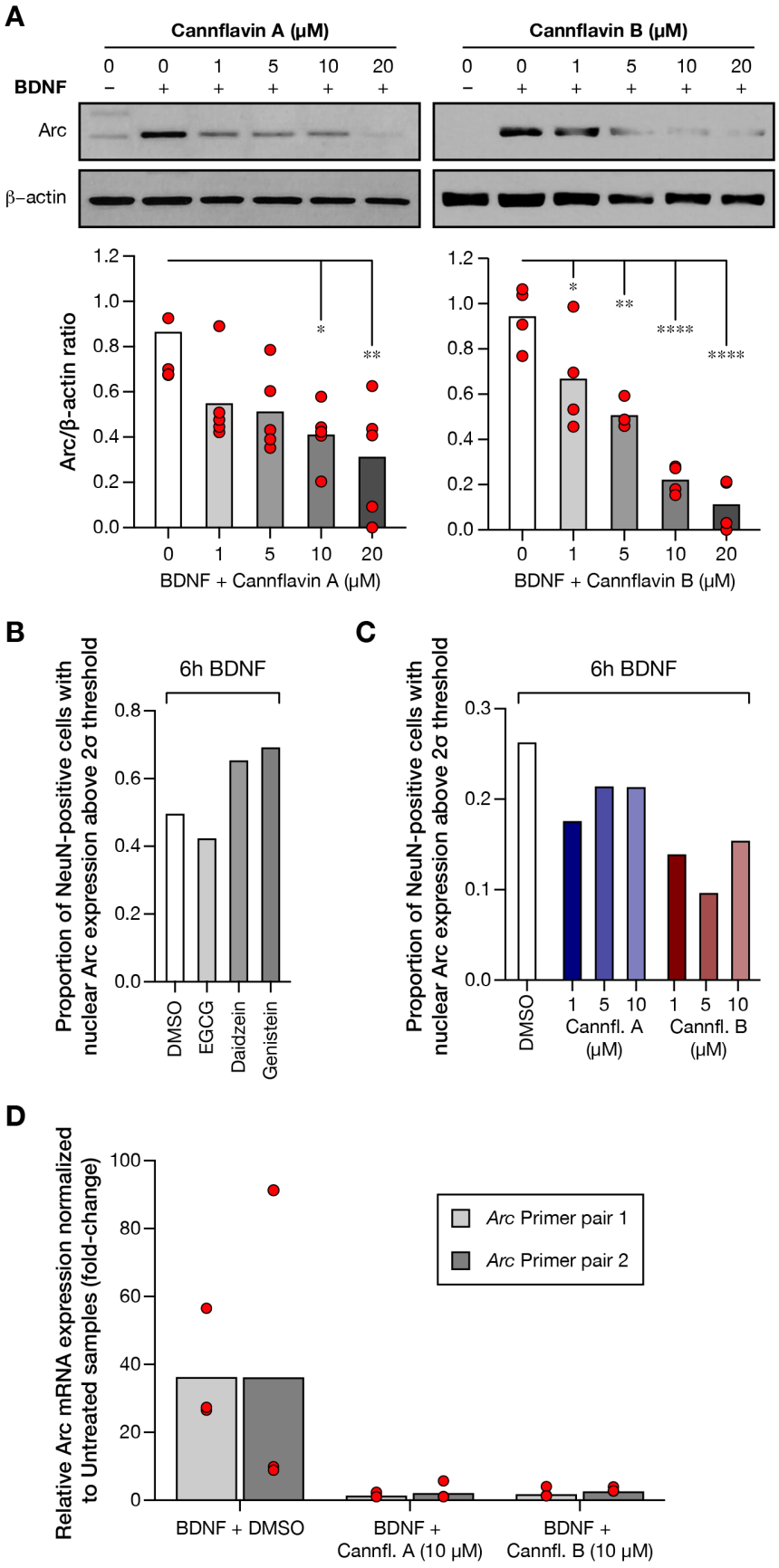


C

Name	Flavanoid class	Structure	Reported impact on BDNF-induced Arc expression in mouse primary cortical neurons at a concentration of 16.7μM (Lalonde et al., 2017)
(–)-Epigallocatechin gallate (EGCG)	Flavanol		**1.9** fold-change (normalized value to BDNF baseline + vehicle treated cells)
Baicalin	Flavone		**1.8** fold-change
Daidzein	Isoflavone		**2.1** fold-change
Genistein	Isoflavone		**1.6** fold-change
7,8-Dihydroxyflavone (7,8-DHF)	Flavone		**1.7** fold-change

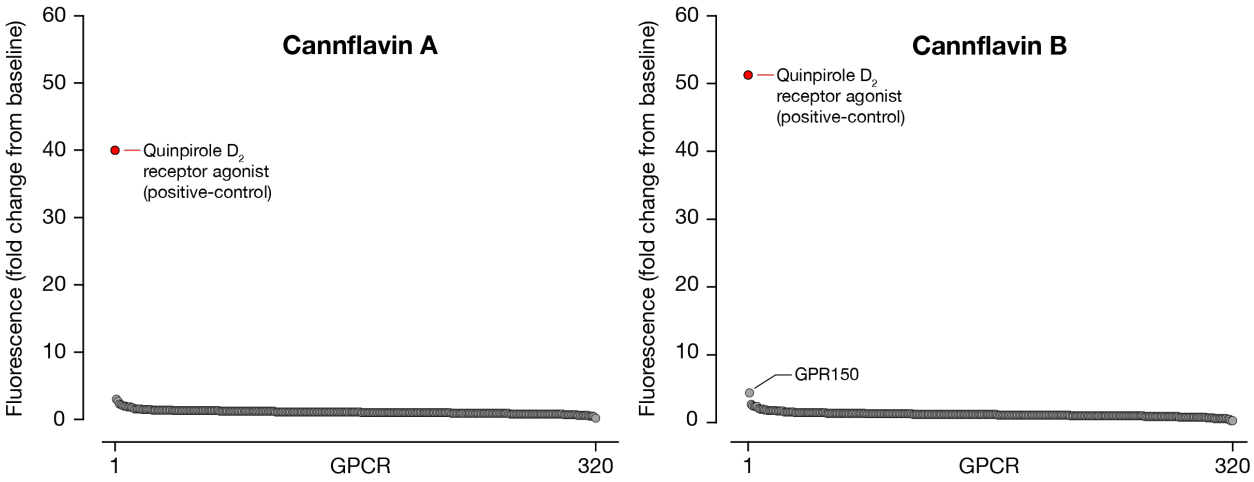
585

586 **Figure 1. Chemical structures of key compounds. A)** Chemical structure for cannflavin A and
587 cannflavin B. **B)** Structure for the small molecule TrkB inhibitor ANA-12. **C)** Table presenting
588 flavonoids organized by name, flavonoid class, chemical structure, and impact of BDNF-induced
589 Arc expression in mouse primary cortical neurons as reported in Lalonde et al. (2017). Position of
590 each ring is labeled for 7,8-dihydroxyflavone.
591
592

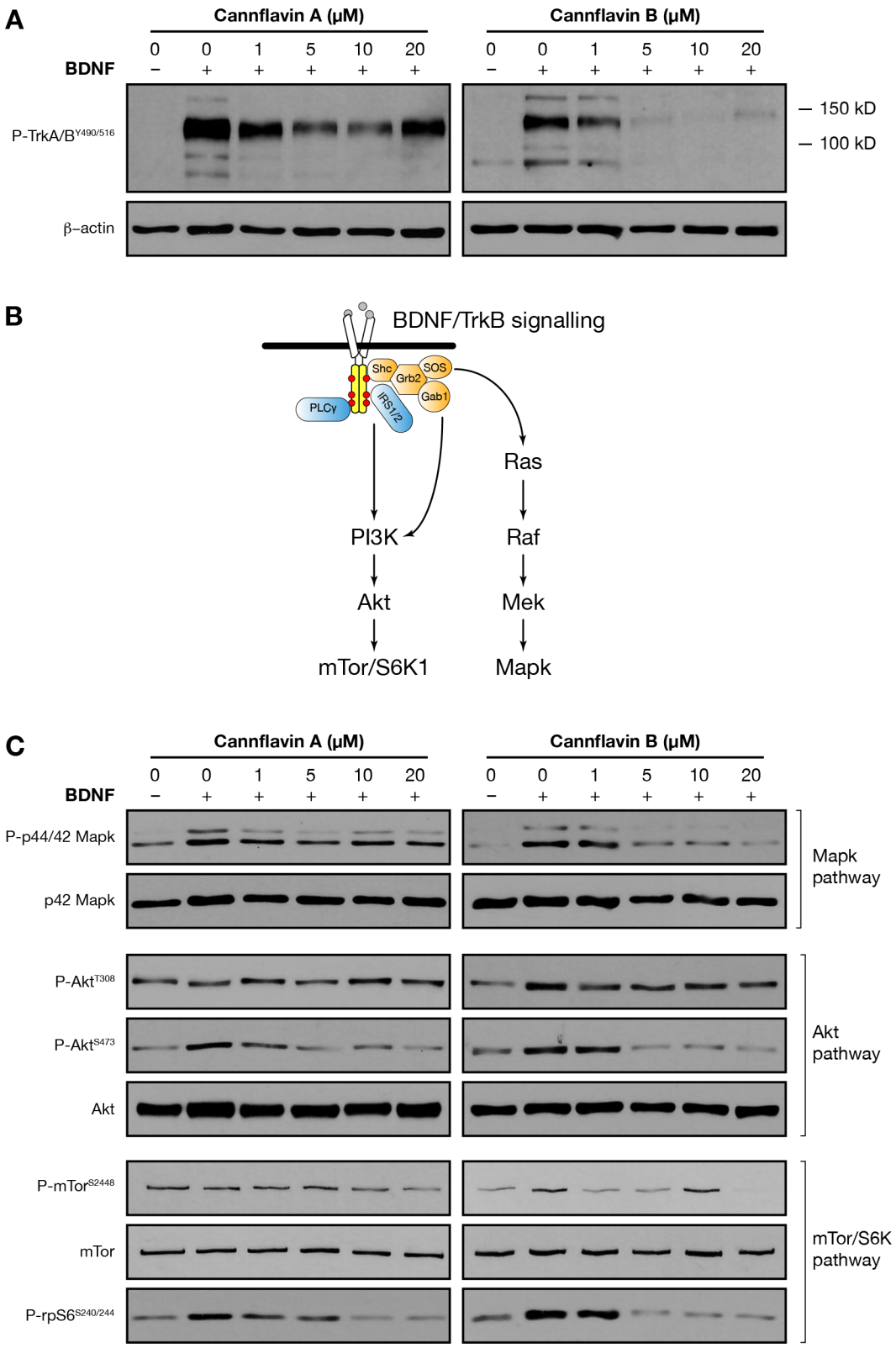

A
Cannflavin A (μM)
Cannflavin B (μM)
0 0 1 5 10 20
BDNF – + + + + +
Arc
β–actin
Arc/β-actin ratio
BDNF + Cannflavin A (μM)
BDNF + Cannflavin B (μM)
B
6h BDNF
Proportion of NeuN-positive cells with nuclear Arc expression above 2σ threshold
DMSO
EGCG
Daidzein
Genistein
C
6h BDNF
Cannfl. A (μM)
Cannfl. B (μM)
D
Relative Arc mRNA expression normalized to Untreated samples (fold-change)
Arc Primer pair 1
Arc Primer pair 2
BDNF + DMSO
BDNF + Cannfl. A (10 μM)
BDNF + Cannfl. B (10 μM)

593

594 **Figure 2. Cannflavin A and cannflavin B decrease BDNF-induced Arc protein and mRNA**
595 **levels in mouse primary cortical neurons. A)** Western blot and corresponding densitometry
596 analysis showing Arc protein abundance in mouse primary cortical cultures when treated with
597 BDNF and various concentrations (0, 1, 5, 10, 20 μM) of cannflavin A (left) or cannflavin B
598 (right). β-actin was used a loading control and graphs show mean of Arc/β-actin ratio for each
599 condition. Biological replicates: $n = 5$ (cannflavin A), $n = 4$ (cannflavin B). One way ANOVA
600 revealed a significant decrease in the abundance of Arc with increasing cannflavin concentrations.
601 Cannflavin A ($F_{4,20} = 4.568$, $p = 0.0088$), Tukeys post-hoc test, * $p < 0.05$, ** $p < 0.001$. Cannflavin
602 B ($F_{4,15} = 24.07$, $p < 0.0001$), Tukeys post-hoc test, * $p < 0.05$, ** $p < 0.001$, **** $p < 0.0001$. **B)**
603 Quantification of immunocytochemistry coverslips treated with various flavonoids (EGCG,
604 daidzein, and genistein; all 10 μM final concentration). Quantification was completed by using a
605 ratio of MAP2-positive cells with nuclear Arc above the 2σ nuclear Arc pixel intensity in the
606 control condition (BDNF treatment alone). **C)** Quantification of immunocytochemistry coverslips
607 treated with various concentrations (0, 1, 5, 10 μM) of cannflavin A (blue bars) or cannflavin B
608 (red bars). Quantification was completed similar to B. **D)** Quantitative real-time PCR *Arc* mRNA
609 analysis using two Arc primer pairs shows a decrease in *Arc* transcripts when treated with 10 μM
610 of cannflavin A or cannflavin B, relative to cells treated with BDNF alone.



611
612
613 **Figure 3. Cannflavins do not stimulate G protein-coupled receptors signaling.** Data for 320
614 GPCRs are presented as an average fold change from baseline upon compound addition.
615 Application of quinpirole to D2 receptor is used as positive control in each plate. Compounds were
616 used at 10 μM and all tests run in quadruplicate.


A
Cannflavin A (μM)
Cannflavin B (μM)
0 0 1 5 10 20
0 0 1 5 10 20
BDNF
– + + + + +
– + + + + +
P-TrkA/B Y490/516
β–actin
150 kD
100 kD
B
BDNF/TrkB signalling
Shc
Grb2
SOS
Gab1
PLCγ
IRS-1/2
Ras
PI3K
Raf
Akt
Mek
mTor/S6K1
Mapk
C
Cannflavin A (μM)
Cannflavin B (μM)
0 0 1 5 10 20
0 0 1 5 10 20
BDNF
– + + + + +
– + + + + +
P-p44/42 Mapk
p42 Mapk
Mapk pathway
P-Akt T308
P-Akt S473
Akt
Akt pathway
P-mTor S2448
mTor
P-rpS6 S240/244
mTor/S6K pathway

617
618
619

620 **Figure 4. Cannflavin A and cannflavin B decrease activation of downstream pathways of**
621 **TrkB. A)** Western blot analysis showing phosphorylation of TrkB when treated with BDNF and
622 various concentrations (0, 1, 5, 10, 20 μM) of cannflavin A (left) or cannflavin B (right). β-actin
623 was used a loading control. **B)** Simplified schematic of BDNF activation of TrkB receptors and
624 downstream signaling pathways. **C)** Western blot analysis showing phosphorylation of Mapk, Akt,
625 and mTor proteins when treated with various concentrations (0, 1, 5, 10, 20 μM) of cannflavin A
626 (left) or cannflavin B (right).

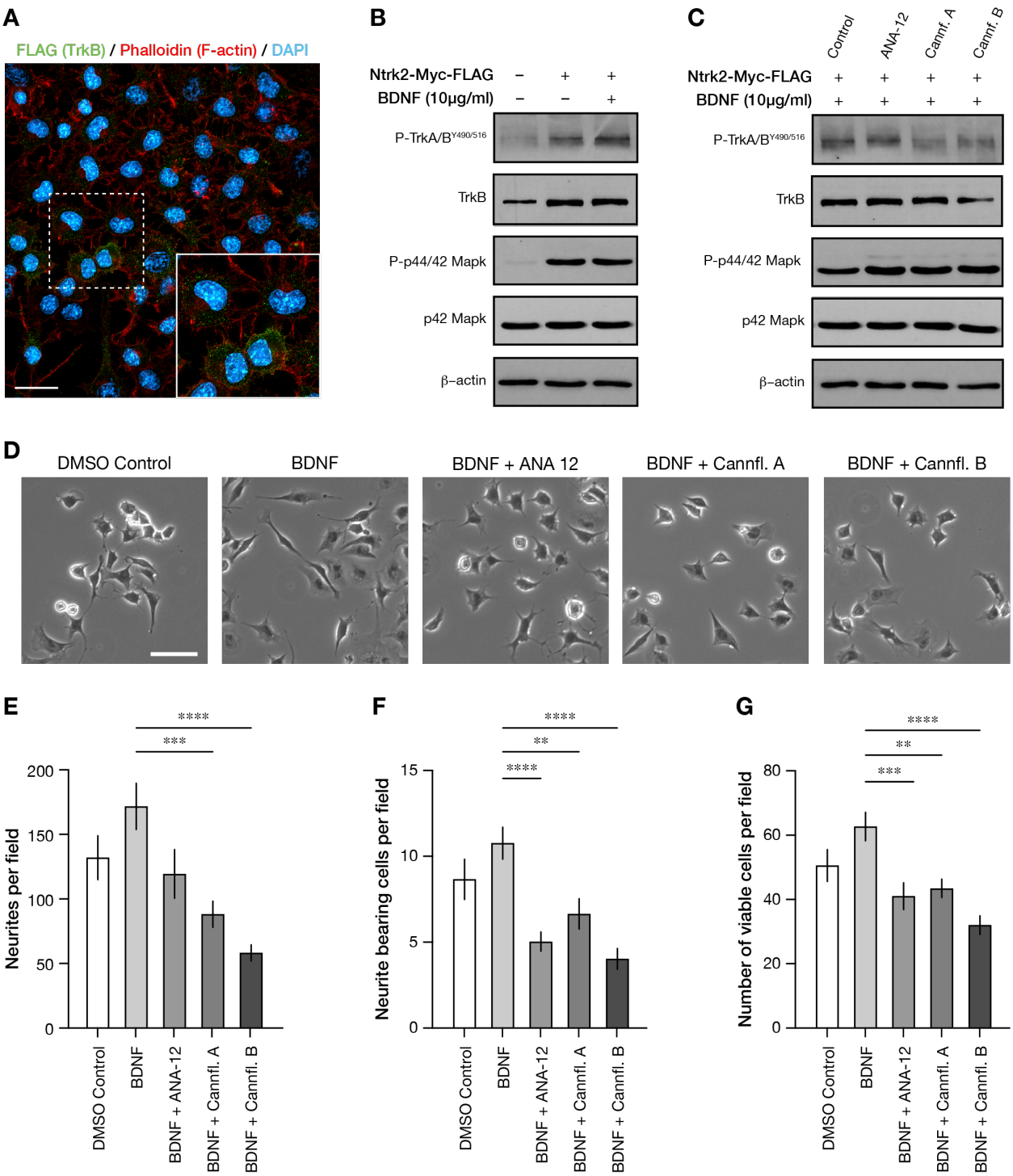


Figure 5. Cannflavin A and cannflavins B reduces BDNF-induced neurite outgrowths in neuroblastoma cells. A) Neuro2a cells were transfected to stably express Ntrk2-Myc-FLAG. Immunocytochemistry was completed to validate that the cells were successfully transfected. Scale

632 bar = 20 μM. **B)** Western blot analysis showing phosphorylation of TrkB and Mapk in regular
633 Neuro2a cells compared to those stably expressing Ntrk2-Myc-FLAG. β-actin was used a loading
634 control. **C)** Western blot analysis showing phosphorylation of TrkB and Mapk when treated with
635 10 μM of ANA-12, cannflavin A, or cannflavin B with the addition of BDNF (10 μg/mL). The
636 small-molecule non-competitive TrkB antagonist ANA-12 was used as a positive control (Cazorla
637 et al., 2011) and β-actin was used as a loading control. **D)** Phase contrast images of Ntrk2-Myc-
638 FLAG Neuro2as treated with or without BDNF (10 μg/mL) and 10 μM of ANA-12, cannflavin A,
639 or cannflavin B. Scale bar = 50 μm. Images were quantified by counting **E)** total number of
640 neurites, and **F)** total number of cells bearing neurites twice the length of cell body, and **G)** number
641 of viable cells per imaged area. One-way ANOVA was used to analyze data. Total number of
642 neurites ($F_{4,150} = 8.264$, $p < 0.001$); total number of cells bearing neurites ($F_{4,150} = 9.433$, $p < 0.001$);
643 number of viable cells ($F_{4,150} = 8.071$, $p < 0.001$). Dunnett's multiple comparisons test to BDNF
644 condition, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *** $p < 0.0001$. Graphs represent mean ±SEM.

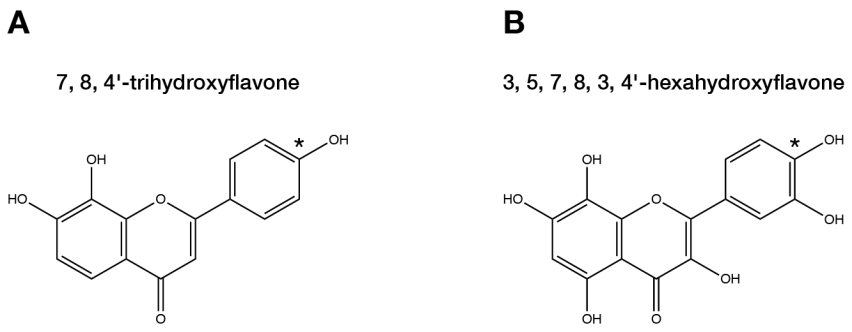
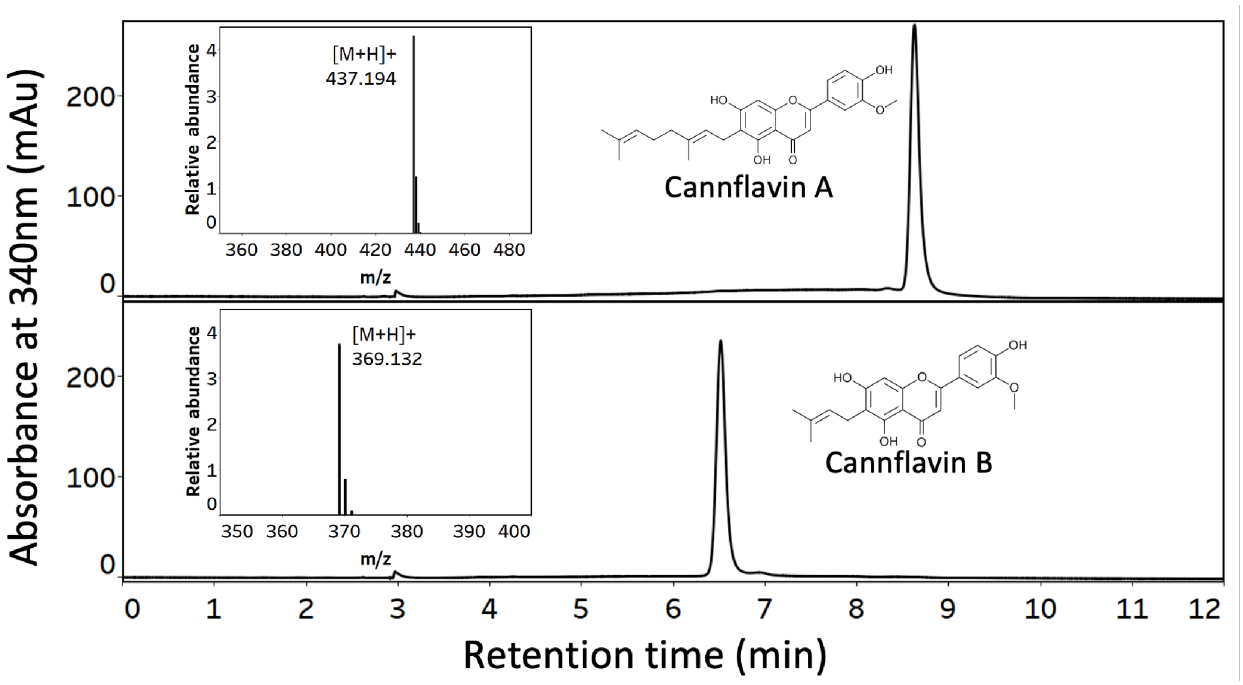


Figure 6. Chemical structure of 7,8-dihydroxyflavone derivatives reported to block TrkB signaling in Liu and colleagues (2010). Asterisk indicates hydroxylated 4' position on B ring of each compound.



Supplementary Figure 1. Enzymatic production of cannflavin A and cannflavin B. Representative chromatograms illustrating pooled fractions of cannflavin A (top) and cannflavin B (bottom) quantified by DAD at 340 nm. Both Q-TOF mass spectra (inset) are consistent with the expected m/z of 6-geranyl chrysoeriol and 6-dimethylallyl chrysoeriol or cannflavins A and B, respectively.

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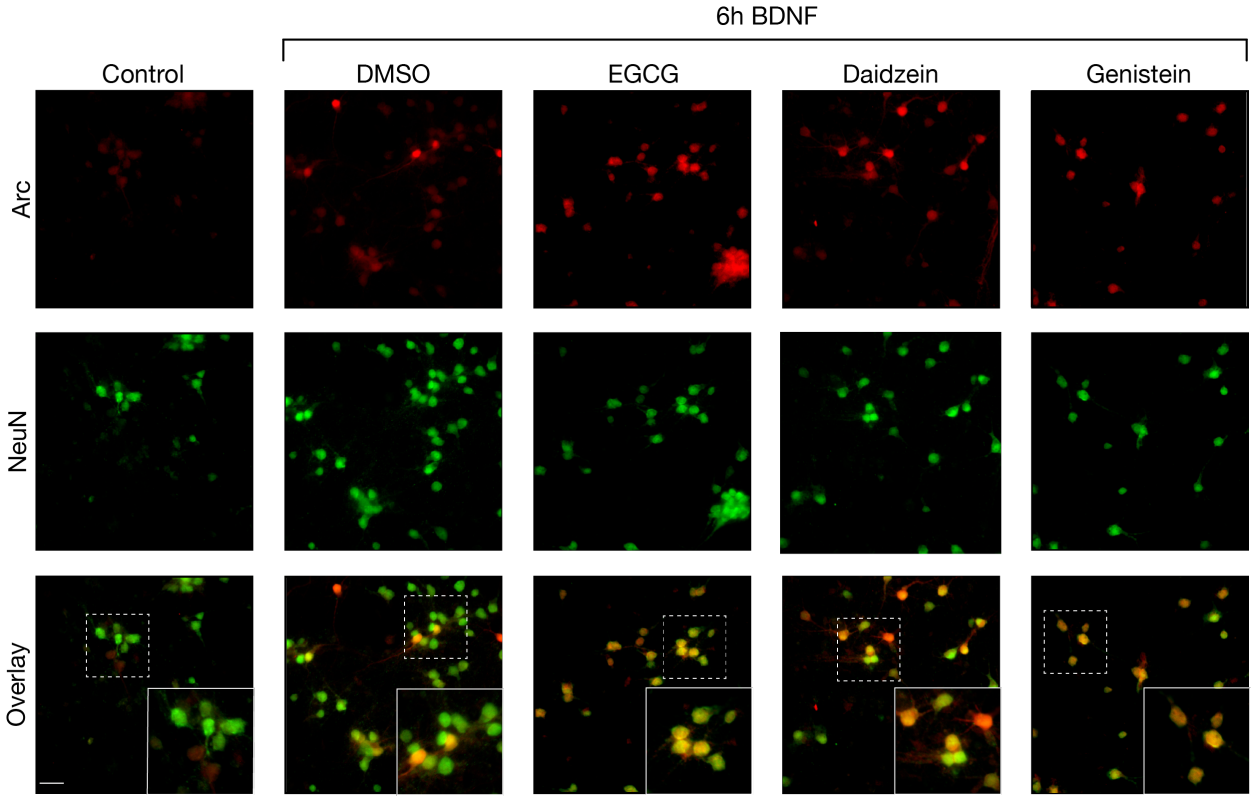

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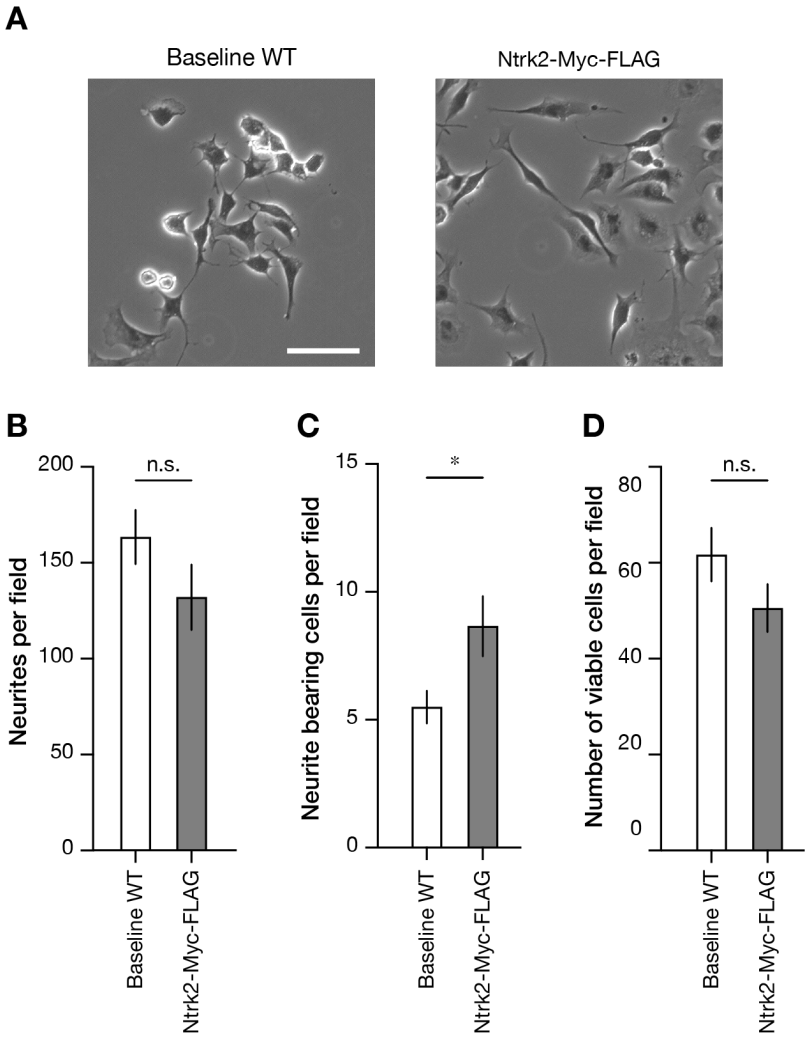
660 **Supplementary Figure 2. Effect of other flavonoids on BDNF-induced Arc expression level**
661 **in mouse primary cortical neurons.** DIV14 cortical neurons were treated with BDNF for 6 h
662 along with ECGC, daidzein, or genistein (final concentration 10 μM). DMSO was used as control.
663 Representative immunostaining of Arc (red fluorophore) in untreated primary cortical neurons and
664 cells that were treated with BDNF and other compounds for 6 h. Consistent with data reported in
665 Lalonde and colleagues (2017), daidzein and genistein increased Arc abundance at the tested
666 concentration. Cells were co-immunostained with the neuronal marker NeuN (green fluorophore)
667 to confirm specificity of staining to neurons. The high-magnification bottom-right insets in each
668 panel show that Arc immunostaining in a BDNF-treated culture is particularly abundant in the
669 nuclear compartment at the 6 h time point. Scale bar = 50 μM.



670

671 **Supplementary Figure 3. Stable overexpression of Myc-FLAG tagged TrkB increases**
672 **neurite outgrowths and survival in Neuro2a cells. A)** Phase contrast images of wild-type (WT,
673 left) and stably overexpressing Ntrk2-Myc-FLAG (right) Neuro2a cells. Images were quantified
674 by counting **B)** total number of neurites, **C)** total number of cells bearing neurites twice the length
675 of cell body, and **D)** number of viable cells per imaged area. * $p < 0.05$, two-tailed unpaired *t*-test.
676 Graphs represent mean ±SEM.

677 **Supplementary Table. GPCRome cannflavins report.** List of all 320 GPCRs tested in the

678 PRESTO-Tango assay.